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Year: 2015

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## Decelerated vascularization in tissue-engineered constructs in association with diabetes mellitus in vivo

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**Abstract:** AIMS: Rapid blood vessel ingrowth in transplanted tissue engineering constructs is the key factor for successful incorporation, but many potential patients who may use engineered tissues suffer from widespread diseases that limit the capacity of neovascularization (e.g. diabetes). Thus, in vivo vascularization analyses of tissue-engineered constructs in angiogenically affected organisms are required. **METHODS:** We therefore investigated the in vivo incorporation of collagen-coated and cell-seeded poly-L-lactide-co-glycolide scaffolds in diabetic B6.BKS(D)-Lepr(db)/J mice using repetitive intravital fluorescence microscopy over a time period of two weeks. For this purpose, scaffolds were seeded with osteoblast-like or bone marrow mesenchymal stem cells and implanted into the dorsal skinfold chambers of diabetic and non-diabetic (C57BL/6) mice. **RESULTS:** Apart from slightly increased inflammatory parameters, diabetic mice showed significantly reduced capillary densities compared with non-diabetic animals from day 6 onward. In line with previous studies, more densely meshed microvascular networks were demonstrated in cell-seeded than in collagen-coated scaffolds from day 6 onward within the single groups (diabetic and control). **CONCLUSIONS:** A large number of patients who suffer from systemic diseases that affect angiogenesis would profit from tissue engineering. Therefore, the challenge for the clinical introduction of tissue-engineered constructs will be to overcome the decreased angiogenesis in diabetic organisms.

DOI: <https://doi.org/10.1016/j.jdiacomp.2015.06.004>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-116514>

Journal Article

Accepted Version



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Originally published at:

Schumann, Paul; Lindhorst, Daniel; Kampmann, Andreas; Gellrich, Nils-Claudius; Krone-Wolf, Sonja; Meyer-Lindenberg, Andrea; von See, Constantin; Gander, Thomas; Lanzer, Martin; Rücker, Martin; Essig, Harald (2015). Decelerated vascularization in tissue-engineered constructs in association with diabetes mellitus in vivo. *Journal of Diabetes and Its Complications*, 29(7):855-864.



# **Decelerated vascularization in tissue-engineered constructs in association with diabetes mellitus in vivo**

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## **Abstract**

**Aims:** Rapid blood vessel ingrowth in transplanted tissue engineering constructs is the key factor for successful incorporation, but many potential patients who may use engineered tissues suffer from widespread diseases that limit the capacity of neovascularization (e.g. diabetes). Thus, *in vivo* vascularization analyses of tissue-engineered constructs in angiogenically affected organisms are required.

**Methods:** We therefore investigated the *in vivo* incorporation of collagen-coated and cell-seeded poly-L-lactide-co-glycolide scaffolds in diabetic B6.BKS(D)-Lepr<sup>db</sup>/J mice using repetitive intravital fluorescence microscopy over a time period of two weeks. For this purpose, scaffolds were seeded with osteoblast-like or bone marrow mesenchymal stem cells and implanted into the dorsal skinfold chambers of diabetic and non-diabetic (C57BL/6) mice.

**Results:** Apart from slightly increased inflammatory parameters, diabetic mice showed significantly reduced capillary densities compared with non-diabetic animals from day 6 onward. In line with previous studies, more densely meshed microvascular networks were demonstrated in cell-seeded than in collagen-coated scaffolds from day 6 onward within the single groups (diabetic and control).

**Conclusions:** A large number of patients who suffer from systemic diseases that affect angiogenesis would profit from tissue engineering. Therefore, the challenge for the clinical introduction of tissue-engineered constructs will be to overcome the decreased angiogenesis in diabetic organisms.

## **Keywords**

Angiogenesis, B6.BKS(D)-Lepr<sup>db</sup>/J mouse, Intravital fluorescence microscopy, PLGA scaffold, Tissue Engineering

## **1. Introduction**

Over the past years, tissue engineering has made considerable progress in the replacement and reconstruction of various tissues. In this process, the vitalization of suitable scaffolds with pluripotent cells (e.g. bone marrow mesenchymal stem cells, bmMSCs) or organ-specific cells (e.g. osteoblast-like cells, OLCs) represents the basic principle [1]. A vital requirement for the successful incorporation of tissue-engineered constructs is rapid vascularization of the transplants. An adequate blood supply is indispensable to satisfy the metabolic demands of the seeded cells concerning the provision of oxygen and nutrients as well as waste disposal [2].

To accelerate vascularization in tissue-engineered constructs seeded with cells, various strategies have been performed, in some cases with remarkable results. Single growth factors, especially vascular endothelial growth factor (VEGF) [3, 4], have been applied as well as combinations of different growth factors [5]. A positive effect on vascularization in tissue-engineered constructs has been demonstrated in co-cultures of different endothelial cell lineages and osteoblasts [6, 7] and in co-cultures of bmMSCs and OLCs [6, 8]. Additional strategies have been generated to prefabricate vascular structures in tissue-engineered constructs [9, 10].

Several of these studies have been conducted using healthy animals. This could be an elementary problem for the transfer into the clinic because many potential patients who may use tissue-engineered constructs suffer from diseases that limit the capacity of neovascularization, e.g. diabetes or hypertonia [11]. In the year 2011, approximately 366 million people suffered from diabetes worldwide. By 2030, this number will have grown to approximately 552 million people (International Diabetes Federation = IDF [12]). Therein, at least 90% are represented by type 2 diabetes [12]. Regarding neovascularization, diabetes plays a special role. On one hand, exorbitant vascularization can be observed (e.g. diabetic

retinopathy and nephropathy), but on the other hand, decreased neovascularization is apparent (e.g. diabetic foot syndrome, reduced wound healing, and graft rejection) [13]. This phenomenon is called the “diabetic paradox” [14]. Concerning the deregulated neovascularization caused by diabetes, more than 100 contributing factors are known [15]. Amongst others, the dysregulation of growth factors such as VEGF, insulin-like growth factor (IGF), and nitric oxide synthase 2 have been described.

Herein, we elucidate directly the *in vivo* vascularization capacity of tissue-engineered constructs in a diabetic organism. For this purpose, poly-L-lactide-co-glycolide (PLGA) scaffolds were seeded with organ-specific cells (OLCs) or undifferentiated progenitor cells (bmMSCs) and implanted into the dorsal skinfold chambers of diabetic B6.BKS(D)-Lepr<sup>db</sup>/J mice. C57BL/6 mice served as controls. After transplantation of the tissue-engineered constructs, we analyzed the angiogenic and inflammatory parameters using repetitive intravital fluorescence microscopy over a time period of two weeks.

## **2. Materials and Methods**

### **2.1 Animals**

The experiments were conducted in accordance with the German legislation for the protection of animals and the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23 Rev. 1985). The experiments were approved by the local governmental animal care committee (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, reference number 33.9-42502-04-08/1437). Male diabetic B6.BKS(D)-Lepr<sup>db</sup>/J mice with a homozygous mutation of the leptin receptor (recessive heredity) were used for the experiments at the age of 13–16 weeks and a body weight of 35–49 g (Jackson Laboratories, Bar Harbor, USA). This mouse strain is characterized by the



development of type 2 diabetes. From the age of two weeks, their level of insulin increases. From the third to fourth week, obesity starts [16] and the blood glucose level begins to rise between the fourth and eighth week [17]. The mice show polyphagia, polydipsia, and polyuria as well as a compensatory hyperplasia of the pancreatic beta cells with hyperinsulinemia [17]. Wound healing is delayed, metabolic efficiency is increased [18, 19], a hypertrophy of the left ventricle is described [16], and the number of blood leukocytes is decreased [20].

Male C57BL/6 (C57Black) mice at the age of 8–15 weeks and a body weight of 18–22 g (WIGA Charles River, Sulzfeld, Germany) were used as controls. This strain represents the genetic background of the B6.BKS(D)-Lepr<sup>db</sup> strain. All animals were housed at one per cage at room temperature (22–24°C) at a relative humidity of 60–65% in a 12 h day–night cycle. The animals had free access to tap water and standard pellet food (Altromin, Lage, Germany) throughout the experiment.

## **2.2 Preparation of the dorsal skinfold chamber**

Both surgery and repetitive intravital fluorescence microscopy were performed under anesthesia by intraperitoneal injection of 0.1 mL of saline solution (Sigma, Steinheim, Germany) per 10 g of body weight. The saline solution contained 90 mg/kg body weight ketamine hydrochloride (Ketavet®; Parke Davis, Freiburg, Germany) and 25 mg/kg body weight dihydroxyldinitrothiazine hydrochloride (Rompun®; Bayer, Leverkusen, Germany).

The dorsal skinfold chamber allowed intravital microscopic surveillance of microcirculation in the anesthetized animal over an extended period. The surgical technique for the preparation of the dorsal skinfold chamber and its implantation have been described previously [21].

PLGA scaffolds were implanted into the dorsal skinfold chamber after a recovery period of a minimum of 48 h subsequent to preparation. For this purpose, animals were anesthetized as described before. The cover slip was removed, and the implant was placed on the striated muscle in the center of the chamber. After the implantation, the chamber was resealed.

### 2.3 Fabrication of tissue-engineered constructs

PLGA scaffolds of  $3 \times 3 \times 1$  mm were fabricated in the Freiburg Institute for Material Research and Macromolecular Chemistry using 3D Bioplotter<sup>TM</sup> operated by the PRIMCAM software (PRIMUS DATA, Einsiedeln, Switzerland) as previously described [22].

To prepare PLGA scaffolds with OLCs, cells were isolated from male C57BL/6 mice aged at 6–8 weeks as presented recently [8]. The mice were euthanized by CO<sub>2</sub>-Inhalation and washed with 70% ethanol (J.T. Baker, Phillipsburg, USA). The frontal and parietal calvaria were removed and sharp dissected free of any adherent tissue. The cleaned bone fragments were kept in HBSS (PAA, Coelbe, Germany) on ice until cell isolation. After mincing bone fragments to pieces of approximately 3x3 mm, cells were isolated using 200 U/ml collagenase II (Cell Systems, St. Katharinen, Germany) in HBSS. Pools of 10 calvarias were digested five times with 5 ml enzyme solution at 37 °C for 10 min with a shaking rate of 150 rpm. The supernatants of digestion 3, 4 and 5 were pooled, pelleted by centrifugation (1200 rpm, 7 min) and washed twice with culture medium (DMEM: 10% fetal calf serum, 20 mM Hepes, 1000 IU/ml penicillin and 0.1 mg/ml streptomycin; PAA, Pasching, Austria). Isolated cells were plated on 100 mm cell culture dishes (Greiner, Frickenhausen, Germany) with DMEM and incubated at 37° C in a humidified incubator with an atmosphere of 91.5% air, 8.5% CO<sub>2</sub> until confluent. Media were changed every 3rd day unless otherwise specified. To prepare secondary subcultures from these cells, the cell layers were gently rinsed with HBSS (PAA) and then incubated with accutase (PAA). Cells released from the culture surface were washed twice with culture medium and aliquoted on new 100 mm cell culture dishes at the desired plating density. Secondary passage cells were characterized by immunocytochemistry.

Bone marrow-derived murine mesenchymal stem cells were isolated according to established protocols based on plastic adherence of these cells [8, 23, 24]. In addition, bone marrow was obtained from male C57BL/6 mice aged at 6–8 weeks. Femurs and tibias were dissected free of surrounding tissue and bone marrow was flushed out with HBSS containing 1000 U/ml

Heparin (Sigma, Steinheim, Germany). The resulting bone marrow was disrupted by passing through a 20-gauge needle, centrifuged at 300 xg for 7 min and resuspended in 3 ml DMEM. The cell suspension was applied to a density gradient (LSM 1077, 1,077g/ml; PAA) [25] and centrifuged again (500 xg, 30 min). The interphase containing bone marrow derived mononuclear cells was transferred to a 15-ml tube (BD Biosciences, Erembodegem, Belgium) and washed three times with 10 ml DMEM (10% FCS). Cells were plated at a density of  $1 \times 10^5$  on cell culture dishes (100 mm; Greiner) and incubated at 37° C in a humidified incubator with an atmosphere of 95% air, 5% CO<sub>2</sub> until confluent. Media were changed every 3rd day unless otherwise specified. Culture medium was MesenCult (StemCell Technologies, Köln, Germany). To prepare secondary subcultures from these cells, the cell layers were gently rinsed with HBSS (PAA) and then incubated with Trypsin/EDTA (PAA). Cells released from the culture surface were washed with two changes of culture medium, and aliquoted on new 100 mm cell culture dishes at the desired plating density. Secondary passage cells were characterized by immunocytochemistry.

For characterization cells were fixed with 4% paraformaldehyd (Sigma) for 10 min. After blocking for 20 min with PBS (Central pharmacy, Hannover Medical School) containing 5% FCS (PAA) and 0.2% Tween (Sigma), cells were incubated with primary antibodies overnight at 4°C and subsequently with secondary antibodies for one hour. Between each step cells were washed three times with PBS for 5min. OLCs were characterized with rabbit anti-mouse collagen I (Biotrend, Köln, Germany), rabbit anti-mouse osteocalcin (Acris Antibodies GmbH, Hiddenhausen, Germany) and rat anti-mouse osteonectin (SPARC, R&D Systems, Wiesbaden, Germany) [26]. Mesenchymal stem cells were characterized with anti-mouse Ly-6A/E (Sca-1) (Biolegend, San Diego, USA) and anti-mouse CD44 (Biolegend) [24]. Cy 2 conjugated goat anti-rabbit antibody and Cy 2 conjugated goat anti-rat antibody (both Dianova, Hamburg, Germany) were used as secondary antibodies. Nuclei were stained using DAPI (Sigma). Cells were examined by fluorescence microscopy (DM4000B Leica Mikrosysteme, Wetzlar,

Germany). For additional characterisation of OLCs, histochemical alkaline phosphatase determination was performed; cells were washed three times with HBSS and incubated for 15 minutes in 5-Bromine-4-Chlorine-3-Indolyl phosphate/nitro blue tetrazolium (BCIP/NBT tablets, Roche, Mannheim, Germany) [27]. Cells were examined by light microscopy (DM4000B Leica Mikrosysteme) without counterstaining.

For *in vivo* experiments, PLGA scaffolds were coated with collagen type I corresponding to standardized guidelines [8]. Cultivated cells were rinsed three times with HBSS and then incubated with accutase (OLCs) or Trypsin/EDTA (bmMSCs) (PAA, Coelbe, Germany) until cells were released from the culture surface. Cells were washed twice with culture medium and counted. Subsequently,  $1 \times 10^4$  OLCs or bmMSCs were resuspended in 10  $\mu$ L of culture medium and spotted onto the PLGA scaffolds coated with collagen type I. The scaffolds with cells were incubated at 37 °C in a humidified incubator in an atmosphere with 91.5% air and 8.5% CO<sub>2</sub> for 4 h to ensure cell attachment to the scaffold surfaces. Scaffolds with attached cells were placed in fresh culture medium and incubated overnight at 37 °C in a humidified incubator in an atmosphere with 95% air and 5% CO<sub>2</sub>. Afterwards, the prepared scaffolds were implanted into the dorsal skinfold chambers of B6.BKS(D)-Lepr<sup>db</sup>/J and C57BL/6 mice.

## 2.4 Intravital fluorescence microscopy

For *in vivo* fluorescence microscopy, mice were anaesthetized and immobilized with a custom-made stereotactic frame (Central Research Laboratory, Hannover Medical School). After intravenous injection of 0.1 mL of 5% fluorescein isothiocyanate (FITC)-labeled dextran 150,000 (Sigma) (for contrast enhancement by staining of blood plasma) and 0.1 mL of 0.1% rhodamine 6G (Sigma) (for direct staining of white blood cells), intravital fluorescence microscopy was performed using a modified Zeiss Axiotech microscope equipped with a 100 W mercury lamp attached to an illuminator with blue, green and ultraviolet filter blocks (Zeiss, Jena, Germany) for epi-illumination. The microscopic images

were recorded by a charge-coupled device video camera (FK-6990, COHU, Prospective Measurements, San Diego, USA) and transferred to a DVD recorder (LQ- MS 800, Panasonic, Hamburg, Germany) for off-line evaluation. With the use of 10x and 20x long distance objectives the images were displayed on a 14 inch video screen (Panasonic, DVD video recorder LQ-MD 800E, Matsushita Electronic Industrial Co Ltd., Osaka, Japan).

## **2.5 Microcirculatory analysis**

Quantitative off-line analysis of the DVDs was performed by means of the computer-assisted image analysis system CapImage (Zeintl, Heidelberg, Germany). Leukocyte-endothelial cell interaction, microhemodynamics and macromolecular leakage were assessed at a magnification of 20x in 4 different microvascular regions of interest (ROIs) in the border zone of the scaffolds. In each ROI, 1-3 venules (inner diameter: 20-40  $\mu\text{m}$ ) were selected for measurements. Leukocytes were classified according to their interaction with the vascular endothelium as adherent, rolling or free-flowing cells as described previously [28]. Adherent leukocytes were defined in each vessel segment as cells that did not move or detach from the endothelial lining within a specified observation period of 20s, and are given as number of cells per square millimeter of endothelial surface, calculated from the diameter and length of the vessel segment studied, assuming a cylindrical vessel geometry [29]. Rolling leukocytes were defined as cells moving with a velocity less than two-fifths of the centerline velocity, and are given as number of cells per minute, passing a reference point within the microvessel. Diameters, centerline velocity, volumetric blood flow, and wall shear rate were determined in those venules in which leukocyte-endothelial cell interaction was analyzed. Diameters ( $d$ ) were measured in  $\mu\text{m}$  perpendicularly to the vessel path. Centerline red blood cell velocity ( $v$ ) was analyzed by the computer-assisted image analysis system using the line shift method. Volumetric blood flow was calculated by  $Q = \pi \times (d/2)^2 \times v/1.6$  (pl/ s), where 1.6 represents the Baker-Wayland factor to correct for the parabolic velocity profile in microvessels with

diameters  $>20\ \mu\text{m}$  [30]. Wall shear rate ( $\gamma$ ) was calculated based on the Newtonian definition:  $\gamma = 8 \times v / d$ . Macromolecular leakage served as an indicator for microvascular permeability and was assessed after an intravenous injection of the macromolecular fluorescent dye FITC-labeled dextran 150.000 by densitometrically determining the grey levels in the tissue directly adjacent to the venular vessel wall ( $E_1$ ), as well as in the marginal cell-free plasma layer within the vessel ( $E_2$ ). Extravasation ( $E$ ) was then calculated as  $E = E_1 / E_2$ .

Angiogenesis was assessed in 200x magnification. For this purpose, functional microvessel density was measured quantitatively in 8 regions of interest (ROI) in the border zone of the scaffolds and in 4 regions of interest (ROI) in the center of the scaffolds. Microvessels which showed a distinct ingrowth, i.e. new capillary buds and sprouts, were counted. By computer assisted analysis using the CapImage software, cumulative length of microvessels per observation area given in  $\text{cm}/\text{cm}^2$  was calculated [31]. For microcirculatory analysis investigators were blinded to the treatment group.

## 2.6 Experimental protocol

The weight of the mice was measured before preparing the dorsal skinfold chamber and every time before performing microcirculatory analysis.

For intravital fluorescence microscopy, 24 B6.BKS(D)-Lepr<sup>db</sup>/J mice were randomly divided into three groups with eight animals in each group. Each group was equipped with dorsal skinfold chambers. The PLGA scaffolds were coated with collagen ( $n = 8$ ), coated with collagen and seeded with OLCs ( $n = 8$ ), or coated with collagen and seeded with bmMSCs ( $n = 8$ ), and then implanted. 24 C57BL/6 mice, also randomly divided into three groups with eight animals in each group, served as controls. During implantation, care was taken to avoid contamination, mechanical disruption, and chamber damage. The macroscopic appearance of the skinfold chamber preparations and the implanted tissue-engineered constructs were documented daily. Intravital fluorescence microscopy of the microhemodynamics, leukocyte-

endothelial cell interaction, macromolecular leakage, and angiogenesis was performed immediately and 3, 6, 10, and 14 days after the implantation.

At the end of the *in vivo* experiments, the animals were killed with an overdose of the anesthetic, and the dorsal skinfold preparations were sacrificed for histological examination.

## **2.7 Histology and immunohistochemistry**

At the end of the *in vivo* experiments, histological examinations of the dorsal skinfold chamber preparations were performed. For light microscopy, formalin-fixed (Merck KGaA, Darmstadt, Germany) specimens of the dorsal skinfold chamber preparations were embedded in paraffin (Leica Mikrosysteme, Wetzlar, Germany). Five-micrometer thick sections were cut and stained with hematoxylin and eosin (HE, both Merck KGaA) according to standard procedures and examined by light microscopy (DM4000B, Leica Mikrosysteme).

For immunohistochemical detection of CD31, as evidence for the presence of endothelial cells, formalin-fixed specimens from the dorsal skinfold chamber were embedded in paraffin and cut into 5- $\mu$ m thick sections. Consecutive sections were incubated with a rabbit anti-mouse CD31 antibody (Acris Antibodies GmbH, Hiddenhausen, Germany). A biotin-conjugated goat anti-rabbit antibody (Dianova, Hamburg, Germany) was used as secondary antibody. Incubation with streptavidin-horseradish peroxidase (Dianova) was followed by color development with aminoethylcarbazole (AEC) substrate (Axxora Deutschland GmbH, Loerrach, Germany) at room temperature. Color development was stopped under microscopic control by washing with water. The sections were counterstained with hemalaun (Merck KGaA) and examined by light microscopy (DM4000B, Leica). For negative control the primary antibody was omitted.

## **2.8 Statistics**

Results are expressed as means  $\pm$  SEM. Differences between groups were assessed by one-way ANOVA and differences within each group were analyzed by one-way ANOVA with repeated measures. To isolate overall differences, appropriate Student-Newman-Keuls or Dunn post-hoc tests were performed. Differences were considered significant at  $p < 0.05$ .

## **3. Results**

### **3.1 Gain / loss of weight**

Significant differences in the gain or loss of weight were not detected in control and diabetic mice throughout the 14-day observation period (data not shown).

### **3.2 Microhemodynamics**

Microhemodynamic parameters were constant and comparable in all experimental groups at any point in time. Values concerning venular diameters, volumetric blood flow, and wall shear rates were measured in postcapillary and collecting venules in direct vicinity to the scaffolds immediately as well as 3, 6, 10, and 14 days after scaffold implantation. The diameters ranged from 20 to 40  $\mu\text{m}$ , and no significant differences could be observed in volumetric blood flow and wall shear rates between the groups throughout the entire experiment (data not shown).

### **3.3 Inflammatory response**

All experimental groups showed a discreet inflammatory response on day 3 after scaffold implantation, represented by a slight increase in rolling and adherent leukocytes as well as a marginal increase in the microvascular permeability, illustrated by the macromolecular



leakage in the postcapillary venules at the border zones of the scaffolds (Fig. 1). The diabetic groups showed slightly higher inflammatory reactions compared with the controls. However, none of the values increased any further, and all values were comparable and constant at any point in time up to day 14 (Fig. 1). For all parameters significant differences could not be detected throughout the entire 14-day observation period.

### **3.4 Angiogenesis and neovascularization**

Typical signs of angiogenesis are characterized by the dilation of capillaries and the formation of capillary buds and sprouts, primarily originating from the venular segments of the striated muscle capillaries and postcapillary venules of the host tissue. These signs of neovascularization could be detected in all experimental groups for the first time on day 3. During the experimental period, these sprouts protruded from both the border zone toward the center of the scaffold and from the center to the border zone, interconnecting with each other and building red blood cell-perfused vascular networks with increased functional capillary densities (Figs. 2 and 3). Thereby the amount of newly formed microvessels was very similar and not significantly different between scaffold centers and border zones within the single groups at any point in time.

Regarding diabetic animals and healthy controls, separate quantitative analysis of functional capillary density demonstrated microvascular networks that were markedly more densely meshed in the vitalized scaffolds than in the collagen-coated PLGA scaffolds from day 6 onward. At any subsequent point in time, the microvascular density of the cell-seeded scaffolds within the diabetic or healthy group was significantly higher compared with that of the collagen-coated scaffolds in the equivalent group. With sole regard to the vitalized scaffolds, there was no significant difference between the scaffolds that were seeded with OLCs or bmMSCs. Throughout the entire 14-day observation period, the extent of capillary

density was comparable within OLC- and bmMSC-seeded scaffolds with reference to the diabetic and non-diabetic groups (Figs. 2 and 3).

Comparing diabetic and healthy mice, the microvascular networks within the single diabetic group (collagen-coated, OLC-seeded, and bmMSC-seeded) showed a very similar time progress compared with the healthy controls. However, from day 6 onward, significantly reduced capillary densities were observed in each case at any point of examination. The trend of reduced microvascular densities was constant between the single diabetic and non-diabetic groups from day 6 up until day 14. With a view of the absolute values at the different moments of examination, the capillary densities of the cell-seeded scaffolds in diabetic mice were approximately comparable to the microvascular networks induced by the scaffolds that were solely coated with collagen in healthy animals (Figs. 2 and 3).

### **3.5 Histology and immunohistochemistry**

To confirm the intravital microscopic results, histology and immunohistochemistry were performed. Sections from paraffin-embedded specimens at day 14 showed noticeable differences in the histological appearance depending on the animal group (diabetic or non-diabetic) and the type of implanted scaffold (Fig. 4). In the collagen-coated scaffolds of diabetic mice, microvascular structures were barely detected. The number of capillary-like structures in the cell-seeded scaffolds in diabetic animals was remarkably higher than that in scaffolds in the absence of seeded cells, and was comparable to the microvasculature of the collagen-coated scaffolds in non-diabetic mice. The microvascular networks of the cell-seeded scaffolds in healthy animals increased distinctly compared with all other scaffolds. Within these two groups, there was no significant difference as to whether the scaffolds were seeded with OLCs or bmMSCs. The identity of the visible vascular structures as microvessels was verified by immunohistochemical detection of the endothelial cell marker CD31 (Fig. 5). Results of the immunohistochemical analysis were congruent with the findings of histology.

#### **4. Discussion**

In the present study, we proved that vascularization decelerated in tissue-engineered constructs in association with diabetes mellitus *in vivo*. After their transplantation into the dorsal skinfold chambers of diabetic B6.BKS(D)-Lepr<sup>db</sup>/J mice, the implants showed a significant decrease in the development of microvascular networks compared with non-diabetic controls (C57BL/6 mice). This observation applied to the scaffolds seeded with organ-specific cells (OLCs) or undifferentiated progenitor cells (bmMSCs) as well as to the non-vitalized scaffolds. The cell-seeded scaffolds showed a significantly higher number of capillary-like structures within their particular group (diabetic and non-diabetic) from day 6 after transplantation, a result which is in line with previous studies [8, 32]. Microhemodynamic parameters were constant in any group and comparable between single groups throughout the entire experiment.

Concerning the selection of an appropriate mouse strain, diabetes can be induced in healthy animals, e.g. by injection of streptozotocin [33]. On the other hand, a strain characterized by genetic development of diabetes can be used. In the present study, mice with genetically induced diabetes were used to prevent the animals from undergoing further invasive examinations (permanent proof of hyperglycemia in mice with medicamentously induced diabetes). Since at least 90% of diabetes in humans are represented by type 2 diabetes [12], the study was performed with a type 2 diabetes strain. Disturbed neoangiogenesis plays a key role in the deranged wound healing in association with diabetes mellitus [13], and rapid vascularization is a basic requirement for the successful incorporation of tissue-engineered constructs [1]. For these reasons, a mouse strain with typical diabetic wound healing deficiency (B6.BKS(D)-Lepr<sup>db</sup>/J) was chosen for the experiments [11, 34]. A potential disadvantage could be the mutation of the leptin receptor. Leptin influences the

proinflammatory immune response [35] as well as T-cell functions [36], which could affect angiogenesis [11, 37].

PLGA scaffolds with a defined pore size of 250  $\mu\text{m}$  have been proven as a well-suited three-dimensional (3D) basic material for tissue-engineered constructs [8, 38]. For *in vivo* microscopic analyses, they were applied in association with the dorsal skinfold chamber because of the excellent comparability with previous investigations [3, 4, 8, 32]. Growth factors were not used in the present study, although several successful applications in diabetic animals have been described [39]. However, the incorporation of growth factors is extremely complicated as it concerns dosage and combinations [40]. Au et al. reported a regression of the microvascular network with the use of angiogenic growth factors in combination with vascular cells [41]. Also, normally proangiogenic nitric oxide showed a converse effect under certain conditions [40]. In particular, special attention must be given to the usage of growth factors in diseases like diabetes, which are characterized by exorbitant vascularization (e.g. diabetic retinopathy) and decreased neovascularization (e.g. diabetic foot syndrome) at the same time (the “diabetic paradox”) [13, 14]. In this context, the medication of growth factors is not terminally clarified [42]. Most remarkably, systemically applied growth factors can induce uncontrolled vessel growth and neoplasms in unexpected regions of the organism [43]. PLGA scaffolds were seeded with OLCs or bmMSCs for different reasons. A typical approach in tissue engineering is the application of organ-specific cells (e.g. OLCs) on one hand and undifferentiated progenitor cells with various differentiation capacities (e.g. bmMSCs) on the other hand. In particular, collagen-coated PLGA scaffolds seeded with OLCs or bmMSCs have been transplanted into the dorsal skinfold chamber of BALB/c mice in a very similar prior investigation [8], which guaranteed excellent comparability. In that study, the accelerated angiogenesis caused by OLCs and bmMSCs was most likely induced by a hypoxia-driven release of VEGF from these cells inside the tissue-engineered constructs rather than a dependence on the potential of bmMSCs to differentiate into specific vascular

cells [8]. Very little is known about OLCs for tissue engineering applications in diabetic animal models, whereas topical administration of mesenchymal cells seeded on collagen scaffolds augmented wound healing and increased angiogenesis in a diabetic rabbit ulcer [44]. Overall, the angiogenic effect of mesenchymal stem cells as a therapeutic target for enhancing diabetic wound healing has been well described [45]. In the current study, OLCs and bmMSCs were isolated and cultivated from the control strain because of various reports on the negative effects on vascularization and wound healing caused by different pluripotent cells of diabetic origin [13, 19].

With regards to the inflammatory host tissue response, diabetic mice and controls showed comparable results to earlier studies concerning the analyzed parameters (rolling and adherent leukocytes, macromolecular leakage) [4, 8, 32]. The moderate increase in these values after implantation of all types of scaffolds at day 3 are in line with other investigations, showing a local transient inflammatory tissue response after implantation of foreign materials [8, 38]. Compared with the controls, the diabetic groups showed slightly higher inflammatory reactions, but without any significant differences. Algenstaedt et al. also demonstrated higher numbers of rolling and adherent leukocytes in a dorsal skinfold chamber model of diabetic mice in comparison with controls [46]. This phenomenon was interpreted by the expression of adhesion molecules induced by glucose. The increased expression of endothelial adhesion molecules is triggered by the excessive production of advanced glycation end products (AGEs) during hyperglycemia [47]. Additionally, AGEs themselves cause an elevated oxidative stress level in endothelial cells [48, 49]. In addition, an ischemia study in diabetic rats showed higher numbers of rolling leukocytes in the mesenteric venules [50]. This result was explained by a reduced volumetric blood flow in the diabetic animals, which could not be detected in the present study. Overall, decreased numbers of blood leukocytes are described for the current diabetic mouse strain compared with healthy control animals [20]. Therefore, it could be assumed that the slight increase in rolling and adherent leukocytes is in fact the

expression of a clearer elevation of these inflammatory parameters. The moderate rise in macromolecular leakage is also confirmed by previous studies with diabetic mice and rats [46, 50], e.g. Martin et al. reported the higher fluorescein permeability of newly formed capillaries in the diabetic retina [13].

Rapid vascularization of tissue-engineered constructs is a vital requirement for their successful incorporation, but many potential users of such constructs suffer from widespread diseases that limit the capacity of neovascularization (e.g. diabetes). As *in vivo* studies of this problem are severely limited, special attention was given to the angiogenesis of the transplanted cell-seeded scaffolds in the diabetic organisms used herein. The chronological development of functional capillary densities in control groups was comparable to a recent study with the same experimental setup using healthy BALB/c mice [8]. Therefore, a conclusion can be drawn with considerable certainty that the accelerated angiogenesis caused by OLCs and bmMSCs in control groups was induced by a hypoxia-driven release of VEGF from the cells. Regarding the diabetic mice, microvascular networks within single groups showed a very similar time progress compared to healthy controls. However, significantly reduced capillary densities were observed from day 6 onward in each case at any point of examination. For that reason, it was concluded that the increased vascularization in cell-seeded scaffolds in diabetic mice was also most likely VEGF-driven based on the hypoxia of the OLCs and bmMSCs in the centers of tissue-engineered constructs. The decrease in vascularization in diabetic animals is in line with other studies. Decelerated neoangiogenesis was reported in the ischemic hindlimb model of diabetic mice [11, 37]. Thereby the possible mechanisms underlying impaired neovascularization in the diabetic organism are highly diverse. Some of these mechanisms involve dysfunctioning and decrease in the number of endothelial progenitor cells [37]. Considerably increased differentiation of MSCs to adipocytes may be another reason for the reduced angiogenic capacity [37]. Concerning diabetic microangiopathy the thickening of the basement membrane has to be considered [49].

Thus migration of cells from the vascular system, which is essential for angiogenesis, is hindered [13]. Lack of VEGF is also responsible for insufficient neovascularization during diabetic wound healing and deranged arteriogenesis [13, 18]. Impaired neovascularization in diabetic mice is associated with decreased concentration of fibroblast growth factor (FGF) [51]. In addition, many other factors are involved in disordered angiogenesis in the diabetic organism, including AGEs, sorbitol, angiopoietin (and its receptors), and nitrogen monoxide, which is crucial for the effects of VEGF, FGF, and transforming growth factor  $\beta$ 1 [13]. The application of VEGF could also expedite vascularization in the ischemic hindlimb model of diabetic mice [52]. However, with regards to the multiple problems associated with the treatment with growth factors [40-43], they were not used in the current investigation.

The presented data demonstrate that the dorsal skinfold chamber represents an excellent model for *in vivo* angiogenesis studies of tissue-engineered constructs, also in diabetic mice. Thus promising research results can be applied in animal models showing impairments, and comparative studies can be performed. For the present study this is of major interest showing significantly decelerated vascularization in diabetic mice compared with that in healthy controls. Moreover, the positive angiogenic effect of seeded OLCs and bmMSCs was most likely VEGF-driven due to hypoxia of the transplanted cells. The challenge for the future will be to compensate for the decreased angiogenesis in tissue-engineered constructs in the diabetic organisms. A hopeful approach could be to transplant tissue-engineered constructs where angiogenesis has already been initiated *in vitro*. Concerning this matter, several promising approaches have been performed recently. Compared with constructs that were not pre-incubated, a two-week pre-incubation period of bmMSC-seeded PLGA scaffolds in Matrigel led to significantly accelerated angiogenesis after transplantation in healthy animals [53]. Among other strategies to generate or prefabricate vascular structures in tissue-engineered constructs by means of microfabrication of vascular networks [9, 10, 54], a culture system promoting the ability of HUVECs (human umbilical vein endothelial cells) to form

capillary-like tube structures has been described [55]. Nevertheless, with a view to the here presented study these auspicious results should be applied in diabetic animal models to obtain reliable evidence.

## **5. Acknowledgements**

The authors are grateful for the excellent technical assistance of Marie Luise Jenzer and Stefanie Rausch.

## **6. Disclosure**

Conflicts of interest: none

## **7. References**

1. Levenberg, S. and R. Langer, *Advances in tissue engineering*. Curr Top Dev Biol, 2004. **61**: p. 113-34.
2. Frerich, B., N. Lindemann, J. Kurtz-Hoffmann, and K. Oertel, *In vitro model of a vascular stroma for the engineering of vascularized tissues*. Int J Oral Maxillofac Surg, 2001. **30**(5): p. 414-20.
3. Kampmann, A., D. Lindhorst, P. Schumann, R. Zimmerer, H. Kokemuller, M. Rucker, N.C. Gellrich, and F. Tavassol, *Additive effect of mesenchymal stem cells and VEGF to vascularization of PLGA scaffolds*. Microvasc Res, 2013. **90**: p. 71-9.
4. Lindhorst, D., F. Tavassol, C. von See, P. Schumann, M.W. Laschke, Y. Harder, K.H. Bormann, H. Essig, H. Kokemuller, A. Kampmann, A. Voss, R. Mulhaupt, M.D. Menger, N.C. Gellrich, and M. Rucker, *Effects of VEGF loading on scaffold-confined vascularization*. J Biomed Mater Res A, 2010. **95**(3): p. 783-92.
5. Richardson, T.P., M.C. Peters, A.B. Ennett, and D.J. Mooney, *Polymeric system for dual growth factor delivery*. Nat Biotechnol, 2001. **19**(11): p. 1029-34.
6. Hofmann, A., U. Ritz, S. Verrier, D. Eglin, M. Alini, S. Fuchs, C.J. Kirkpatrick, and P.M. Rommens, *The effect of human osteoblasts on proliferation and neo-vessel formation of human umbilical vein endothelial cells in a long-term 3D co-culture on polyurethane scaffolds*. Biomaterials, 2008. **29**(31): p. 4217-26.
7. Santos, M.I., R.E. Unger, R.A. Sousa, R.L. Reis, and C.J. Kirkpatrick, *Crosstalk between osteoblasts and endothelial cells co-cultured on a polycaprolactone-starch scaffold and the in vitro development of vascularization*. Biomaterials, 2009. **30**(26): p. 4407-15.
8. Schumann, P., F. Tavassol, D. Lindhorst, C. Stuehmer, K.H. Bormann, A. Kampmann, R. Mulhaupt, M.W. Laschke, M.D. Menger, N.C. Gellrich, and M. Rucker, *Consequences of*



- seeded cell type on vascularization of tissue engineering constructs in vivo*. Microvascular Research, 2009. **78**(2): p. 180-190.
9. Shin, M., K. Matsuda, O. Ishii, H. Terai, M. Kaazempur-Mofrad, J. Borenstein, M. Detmar, and J.P. Vacanti, *Endothelialized networks with a vascular geometry in microfabricated poly(dimethyl siloxane)*. Biomedical Microdevices, 2004. **6**(4): p. 269-278.
10. Rubenstein, D., D. Han, S. Goldgraben, E. El-Gendi, P.I. Gouma, and M.D. Frame, *Bioassay chamber for angiogenesis with perfused explanted arteries and electrospun scaffolding*. Microcirculation, 2007. **14**(7): p. 723-737.
11. Schiekofer, S., G. Galasso, K. Sato, B.J. Kraus, and K. Walsh, *Impaired revascularization in a mouse model of type 2 diabetes is associated with dysregulation of a complex angiogenic-regulatory network*. Arteriosclerosis Thrombosis and Vascular Biology, 2005. **25**(8): p. 1603-1609.
12. Schumann, P., D. Lindhorst, M.E. Wagner, A. Schramm, N.C. Gellrich, and M. Rucker, *Perspectives on resorbable osteosynthesis materials in craniomaxillofacial surgery*. Pathobiology, 2013. **80**(4): p. 211-7.
13. Martin, A., M.R. Komada, and D.C. Sane, *Abnormal angiogenesis in diabetes mellitus*. Med Res Rev, 2003. **23**(2): p. 117-45.
14. Tepper, O.M., R.D. Galiano, J.M. Capla, C. Kalka, P.J. Gagne, G.R. Jacobowitz, J.P. Levine, and G.C. Gurtner, *Human endothelial progenitor exhibit impaired proliferation, cells from type II diabetics adhesion, and incorporation into vascular structures*. Circulation, 2002. **106**(22): p. 2781-2786.
15. Brem, H. and M. Tomic-Canic, *Cellular and molecular basis of wound healing in diabetes*. Journal of Clinical Investigation, 2007. **117**(5): p. 1219-1222.
16. Barouch, L.A., D.E. Berkowitz, R.W. Harrison, C.P. O'Donnell, and J.M. Hare, *Disruption of leptin signaling contributes to cardiac hypertrophy independently of body weight in mice*. Circulation, 2003. **108**(6): p. 754-9.
17. Bates, S.H., W.H. Stearns, T.A. Dundon, M. Schubert, A.W. Tso, Y. Wang, A.S. Banks, H.J. Lavery, A.K. Haq, E. Maratos-Flier, B.G. Neel, M.W. Schwartz, and M.G. Myers, Jr., *STAT3 signalling is required for leptin regulation of energy balance but not reproduction*. Nature, 2003. **421**(6925): p. 856-9.
18. Trousdale, R.K., S. Jacobs, D.A. Simhaee, J.K. Wu, and J.W. Lustbader, *Wound closure and metabolic parameter variability in a db/db mouse model for diabetic ulcers*. J Surg Res, 2009. **151**(1): p. 100-7.
19. Stepanovic, V., O. Awad, C. Jiao, M. Dunnwald, and G.C. Schatteman, *Leprdb diabetic mouse bone marrow cells inhibit skin wound vascularization but promote wound healing*. Circ Res, 2003. **92**(11): p. 1247-53.
20. Lu, F.L., R.A. Johnston, L. Flynt, T.A. Theman, R.D. Terry, I.N. Schwartzman, A. Lee, and S.A. Shore, *Increased pulmonary responses to acute ozone exposure in obese db/db mice*. Am J Physiol Lung Cell Mol Physiol, 2006. **290**(5): p. L856-65.
21. Lehr, H.A., M. Leunig, M.D. Menger, D. Nolte, and K. Messmer, *Dorsal skinfold chamber technique for intravital microscopy in nude mice*. Am J Pathol, 1993. **143**(4): p. 1055-62.
22. Schumann, P., H. Kokemuller, F. Tavassol, D. Lindhorst, J. Lemound, H. Essig, M. Rucker, and N.C. Gellrich, *Optic nerve monitoring*. Craniomaxillofac Trauma Reconstr, 2013. **6**(2): p. 75-86.
23. Lennon, D.P. and A.I. Caplan, *Isolation of rat marrow-derived mesenchymal stem cells*. Exp Hematol, 2006. **34**(11): p. 1606-7.
24. Meirelles Lda, S. and N.B. Nardi, *Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization*. Br J Haematol, 2003. **123**(4): p. 702-11.
25. Aurich, I., L.P. Mueller, H. Aurich, J. Luetzkendorf, K. Tisljar, M.M. Dollinger, W. Schormann, J. Walldorf, J.G. Hengstler, W.E. Fleig, and B. Christ, *Functional integration of hepatocytes derived from human mesenchymal stem cells into mouse livers*. Gut, 2007. **56**(3): p. 405-15.
26. Choi, J.Y., B.H. Lee, K.B. Song, R.W. Park, I.S. Kim, K.Y. Sohn, J.S. Jo, and H.M. Ryoo, *Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3-E1 cells*. J Cell Biochem, 1996. **61**(4): p. 609-18.
27. Park, B.W., Y.S. Hah, M.J. Choi, Y.M. Ryu, S.G. Lee, D.R. Kim, J.R. Kim, and J.H. Byun, *In vitro osteogenic differentiation of cultured human dental papilla-derived cells*. J Oral Maxillofac Surg, 2009. **67**(3): p. 507-14.
28. Rucker, M., T. Schafer, F. Roesken, W.J. Spitzer, M. Bauer, and M.D. Menger, *Reduction of inflammatory response in composite flap transfer by local stress conditioning-induced heat-shock protein 32*. Surgery, 2001. **129**(3): p. 292-301.

29. Laschke, M.W., K. Witt, T. Pohlemann, and M.D. Menger, *Injectable nanocrystalline hydroxyapatite paste for bone substitution: in vivo analysis of biocompatibility and vascularization*. J Biomed Mater Res B Appl Biomater, 2007. **82**(2): p. 494-505.
30. Baker, M. and H. Wayland, *On-line volume flow rate and velocity profile measurement for blood in microvessels*. Microvasc Res, 1974. **7**(1): p. 131-43.
31. Menger, M.D., D. Steiner, and K. Messmer, *Microvascular ischemia-reperfusion injury in striated muscle: significance of "no reflow"*. Am J Physiol, 1992. **263**(6 Pt 2): p. H1892-900.
32. Schumann, P., C. von See, A. Kampmann, D. Lindhorst, F. Tavassol, H. Kokemuller, K.H. Bormann, N.C. Gellrich, and M. Rucker, *Comparably accelerated vascularization by preincorporation of aortic fragments and mesenchymal stem cells in implanted tissue engineering constructs*. J Biomed Mater Res A, 2011. **97**(4): p. 383-94.
33. Arora, S., S.K. Ojha, and D. Vohora, *Characterisation of streptozotocin induced diabetes mellitus in Swiss albino mice*. Global J Pharmacol, 2009. **3**(2): p. 81-84.
34. Galeano, M., V. Torre, B. Deodato, G.M. Campo, M. Colonna, A. Sturiale, F. Squadrito, V. Cavallari, D. Cucinotta, M. Buemi, and D. Altavilla, *Raxofelast, a hydrophilic vitamin E-like antioxidant, stimulates wound healing in genetically diabetic mice*. Surgery, 2001. **129**(4): p. 467-77.
35. Loffreda, S., S.Q. Yang, H.Z. Lin, C.L. Karp, M.L. Brengman, D.J. Wang, A.S. Klein, G.B. Bulkley, C. Bao, P.W. Noble, M.D. Lane, and A.M. Diehl, *Leptin regulates proinflammatory immune responses*. FASEB J, 1998. **12**(1): p. 57-65.
36. Martin-Romero, C., J. Santos-Alvarez, R. Goberna, and V. Sanchez-Margalet, *Human leptin enhances activation and proliferation of human circulating T lymphocytes*. Cell Immunol, 2000. **199**(1): p. 15-24.
37. Yan, J., G. Tie, B. Park, Y. Yan, P.T. Nowicki, and L.M. Messina, *Recovery from hind limb ischemia is less effective in type 2 than in type 1 diabetic mice: roles of endothelial nitric oxide synthase and endothelial progenitor cells*. Journal of vascular surgery, 2009. **50**(6): p. 1412-1422.
38. Rücker, M., M.W. Laschke, D. Junker, C. Carvalho, A. Schramm, R. Mülhaupt, N.-C. Gellrich, and M.D. Menger, *Angiogenic and inflammatory response to biodegradable scaffolds in dorsal skinfold chambers of mice*. Biomaterials, 2006. **27**(29): p. 5027-5038.
39. Galeano, M., D. Altavilla, D. Cucinotta, G.T. Russo, M. Calo, A. Bitto, H. Marini, R. Marini, E.B. Adamo, P. Seminara, L. Minutoli, V. Torre, and F. Squadrito, *Recombinant human erythropoietin stimulates angiogenesis and wound healing in the genetically diabetic mouse*. Diabetes, 2004. **53**(9): p. 2509-17.
40. Phelps, E.A. and A.J. Garcia, *Update on therapeutic vascularization strategies*. Regen Med, 2009. **4**(1): p. 65-80.
41. Au, P., J. Tam, D.G. Duda, P.-C. Lin, L.L. Munn, D. Fukumura, and R.K. Jain, *Paradoxical Effects of PDGF-BB Overexpression in Endothelial Cells on Engineered Blood Vessels*. The American journal of pathology, 2009. **175**(1): p. 294-302.
42. Pardue, E.L., S. Ibrahim, and A. Ramamurthi, *Role of hyaluronan in angiogenesis and its utility to angiogenic tissue engineering*. Organogenesis, 2008. **4**(4): p. 203-214.
43. Ferrara, N. and T. Davis-Smyth, *The biology of vascular endothelial growth factor*. Endocr Rev, 1997. **18**(1): p. 4-25.
44. O'Loughlin, A., M. Kulkarni, M. Creane, E.E. Vaughan, E. Mooney, G. Shaw, M. Murphy, P. Dockery, A. Pandit, and T. O'Brien, *Topical administration of allogeneic mesenchymal stromal cells seeded in a collagen scaffold augments wound healing and increases angiogenesis in the diabetic rabbit ulcer*. Diabetes, 2013. **62**(7): p. 2588-94.
45. Gu, C., S. Huang, D. Gao, Y. Wu, J. Li, K. Ma, X. Wu, and X. Fu, *Angiogenic Effect of Mesenchymal Stem Cells as a Therapeutic Target for Enhancing Diabetic Wound Healing*. The international journal of lower extremity wounds, 2014: p. 1534734614534977.
46. Algenstaedt, P., C. Schaefer, T. Biermann, A. Hamann, B. Schwarzloh, H. Greten, W. Ruther, and N. Hansen-Algenstaedt, *Microvascular alterations in diabetic mice correlate with level of hyperglycemia*. Diabetes, 2003. **52**(2): p. 542-9.
47. Hadi, H.A. and J.A. Suwaidi, *Endothelial dysfunction in diabetes mellitus*. Vasc Health Risk Manag, 2007. **3**(6): p. 853-76.
48. Hunt, J.V., R.T. Dean, and S.P. Wolff, *Hydroxyl radical production and autoxidative glycosylation. Glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing*. Biochem J, 1988. **256**(1): p. 205-12.
49. Orasanu, G. and J. Plutzky, *The pathologic continuum of diabetic vascular disease*. J Am Coll Cardiol, 2009. **53**(5 Suppl): p. S35-42.

50. Panes, J., I. Kurose, D. Rodriguez-Vaca, D.C. Anderson, M. Miyasaka, P. Tso, and D.N. Granger, *Diabetes exacerbates inflammatory responses to ischemia-reperfusion*. Circulation, 1996. **93**(1): p. 161-7.
51. Takeuchi, K., K. Takehara, K. Tajima, S. Kato, and T. Hirata, *Impaired healing of gastric lesions in streptozotocin-induced diabetic rats: effect of basic fibroblast growth factor*. J Pharmacol Exp Ther, 1997. **281**(1): p. 200-7.
52. Galiano, R.D., O.M. Tepper, C.R. Pelo, K.A. Bhatt, M. Callaghan, N. Bastidas, S. Bunting, H.G. Steinmetz, and G.C. Gurtner, *Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells*. Am J Pathol, 2004. **164**(6): p. 1935-47.
53. Schumann, P., D. Lindhorst, C. von See, N. Menzel, A. Kampmann, F. Tavassol, H. Kokemuller, M. Rana, N.C. Gellrich, and M. Rucker, *Accelerating the early angiogenesis of tissue engineering constructs in vivo by the use of stem cells cultured in matrigel*. J Biomed Mater Res A, 2014. **102**(6): p. 1652-62.
54. Fidkowski, C., M.R. Kaazempur-Mofrad, J. Borenstein, J.P. Vacanti, R. Langer, and Y.D. Wang, *Endothelialized microvasculature based on a biodegradable elastomer*. Tissue Engineering, 2005. **11**(1-2): p. 302-309.
55. Sala, A., P. Hanseler, A. Ranga, M.P. Lutolf, J. Voros, M. Ehrbar, and F.E. Weber, *Engineering 3D cell instructive microenvironments by rational assembly of artificial extracellular matrices and cell patterning*. Integr Biol (Camb), 2011. **3**(11): p. 1102-11.

## **8. Figure legends**

Fig. 1: Inflammatory response of the host tissue caused by the implantation of tissue-engineered constructs. Number of rolling leukocytes, given as cells/min (A), number of adherent leukocytes, given as cells/mm<sup>2</sup> (B), and macromolecular leakage as an indicator of microvascular permeability (C) in postcapillary and collecting venules within the border zones of collagen-coated (controls: white bars; diabetic mice: white bars, hatched) PLGA scaffolds, additionally seeded with OLCs (controls: gray bars; diabetic mice: gray bars, hatched) or bmMSCs (controls: dark gray bars; diabetic mice: dark gray bars, hatched) immediately (day 0) as well as 3, 6, 10, and 14 days after implantation into the dorsal skinfold chamber of BALB/c mice (any group, n=8). Means  $\pm$  SEM.

Fig. 2: Microvascular networks 6 days after the implantation (scaffold center: A and D; border zone: B, C, E, and F) in C57BL/6 (A, B, and C) and diabetic mice (D, E, and F). Intravital fluorescence microscopy of collagen-coated scaffolds (A and D), additionally seeded with OLCs (B and E) or bmMSCs (C and F). Note the considerably more densely meshed

microvascular networks in C57BL/6 mice. Blue light epi-illumination with contrast enhancement by addition of 5% FITC-labeled dextran 150,000. PLGA strands are marked with asterisks.

Fig. 3: Time course of functional microvascular densities. Quantitative assessment of the functional microvascular density of newly formed microvessels, given in  $\text{cm}/\text{cm}^2$ , of collagen-coated (controls: white bars; diabetic mice: white bars, hatched) PLGA scaffolds, additionally seeded with OLCs (controls: gray bars; diabetic mice: gray bars, hatched) or bmMSCs (controls: dark gray bars; diabetic mice: dark gray bars, hatched) immediately (day 0) as well as 3, 6, 10, and 14 days after implantation into the dorsal skinfold chamber of BALB/c mice (any group,  $n=8$ ). Means  $\pm$  SEM. \* $p < 0.05$  versus controls with collagen-coated scaffolds and all diabetic groups; ° $p < 0.05$  versus diabetic mice with collagen-coated scaffolds; + $p < 0.05$  versus the same group on the previous day.

Fig. 4: Histological appearance of paraffin-embedded specimens 14 days after the implantation into the skinfold chamber of BALB/c mice. HE staining of collagen-coated (controls: A and B; diabetic mice: G and H) PLGA scaffolds, additionally seeded with OLCs (controls: C and D; diabetic mice: I and J) or bmMSCs (controls: E and F; diabetic mice: K and L). Low magnification pictures (left column: A, C, E, G, I, and K) provide an overview of the gross appearance of the scaffolds. After 14 days, the scaffold strands (asterisks) were embedded in the vascularized granulation tissue. The differences in microvascular densities are clarified by higher magnification pictures (right column: B, D, F, H, J, and L). Arrows denote the vascular structures.

Fig. 5: Immunohistochemical detection of CD31 as a marker of endothelial cells (arrows) in collagen-coated (controls: A and B; diabetic mice: G and H) PLGA scaffolds, additionally

seeded with OLCs (controls: C and D; diabetic mice: I and J) or bmMSCs (controls: E and F; diabetic mice: K and L). At day 14, a positive reaction for CD31 was detectable in all constructs, but marked differences in vascular density were observed depending on the composition of the constructs. PLGA strands are marked with asterisks. Left column (A, C, E, G, I, and K): overview, right column (B, D, F, H, J, and L): higher magnification.